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Identification of the motifs within the tobacco mosaic virus 5′-leader responsible for enhancing translation

Daniel R.Gallie and Virginia Walbot[1]
Department of Biochemistry, University of California, Riverside, CA 92521-0129 and [1]Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020, USA

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ABSTRACT

The leader (called Ω) of tobacco mosaic virus RNA enhances translation in both eukaryotes and prokaryotes. Although little secondary structure is predicted to exist within Ω, the primary sequence of the 68 base leader is highly organized. Three copies of an eight base direct repeat and a $(CAA)_n$ region represent the two motifs found in the leaders of many TMV strains, and together these comprise 72% of Ω. In previous deletion studies, no mutants exhibited loss-of-function, suggesting that functional redundancy exists within Ω. We report here that a more comprehensive deletion analysis identified the motifs involved in translational enhancement. In a separate approach, oligonucleotides containing the sequence of each motif were used to construct leaders that varied in the number and configuration of the motifs. β-Glucuronidase mRNA constructs containing these mutant leaders were synthesized *in vitro* and their translational efficiency measured *in vivo* following mRNA delivery to carrot protoplasts via electroporation. A combination of one copy of the 8 base direct repeat and a 25 base $(CAA)_n$ region was identified as the core regulatory element, although the $(CAA)_n$ motif is more critical. Two copies of the $(CAA)_n$ region are sufficient to confer a high level of enhancement and a leader composed of multiple copies of the direct repeat is moderately enhancing. Thus, these two motifs are functionally redundant.

INTRODUCTION

The genomic RNA of tobacco mosaic virus (TMV) is 6394 bases in length and functions as an mRNA for the replicase protein (1). Although TMV RNA does not terminate in a poly(A) tail, the RNA is efficiently translated. Analysis of chimeric mRNAs has demonstrated that Ω, the 68 base leader of TMV (2), enhances translation *in vivo* (3,4,5) and *in vitro* (6,7). As Ω does not affect transcript stability, it specifically operates as a regulator of translation (3,5). As such, Ω is functionally dependent on both the ribosome source and ribosome concentration (8). The degree to which Ω enhances translation is highly species-dependent. Enhancement is greatest in dicotyledonous plant cells (3), moderate in *Xenopus* oocytes (7), cultured mammalian cells (5), and monocotyledonous cells (3), and not detectable in yeast (J.Everett and D.Gallie, manuscript submitted).

Ω from the U1 strain of TMV is a highly organized sequence. Two motifs, a reiterated eight base direct repeat and a 25 base $(CAA)_n$ region, make up 72% of the leader. Although the leaders from other TMV strains vary in length, similar elements are present in all strains (Fig. 1). Given that the sequence of a leader is not subject to the same selective pressure as the coding region, we thought it possible that these TMV leader elements may play a role in either the translational enhancement and/or in viral RNA replication. A conservative deletion analysis, in which only one copy of a motif was deleted at a time, did not result in any substantial loss-of-function mutants (9). The replacement of the $(CAA)_n$ region with a poly(U) sequence did result in loss of translational enhancement, however, the $(CAA)_n$ region was not thought essential at the time as deletion of this motif resulted in a leader that was 52% as active as Ω itself (9). One conclusion from these experiments was that functional redundancy existed within Ω.

In this report, we demonstrate that a more encompassing deletion analysis of Ω did generate loss-of-function mutants. Analysis of these mutants suggested that the core regulatory element responsible for the enhancement is composed of one copy of each motif with the $(CAA)_n$ region being the most critical. An alternative and complementary approach, in which oligonucleotides representing the motifs were used to construct leaders varying in motif number and content, resulted in the same conclusion. These results support the hypothesis that the primary structure of Ω does play an active role in regulating translational efficiency.

MATERIAL AND METHODS

mRNA leader constructs and in vitro transcription reaction conditions

Oligonucleotides representing Ω and Ω-deletion mutants were synthesized using an Applied Biosystems 380B DNA Synthesizer. The appropriate complementary oligonucleotides were annealed before introduction into pT7-based vector, pT7-GUS-A_{25} (3). The presence of a poly$(A)_{25}$ tract downstream of the GUS coding region allowed generation of poly$(A)^+$ GUS mRNA as previously described (5). For the M-series, pT7-GUS-A_{25} was modified to introduce the sequence, 5′-GTATTTT-3′,

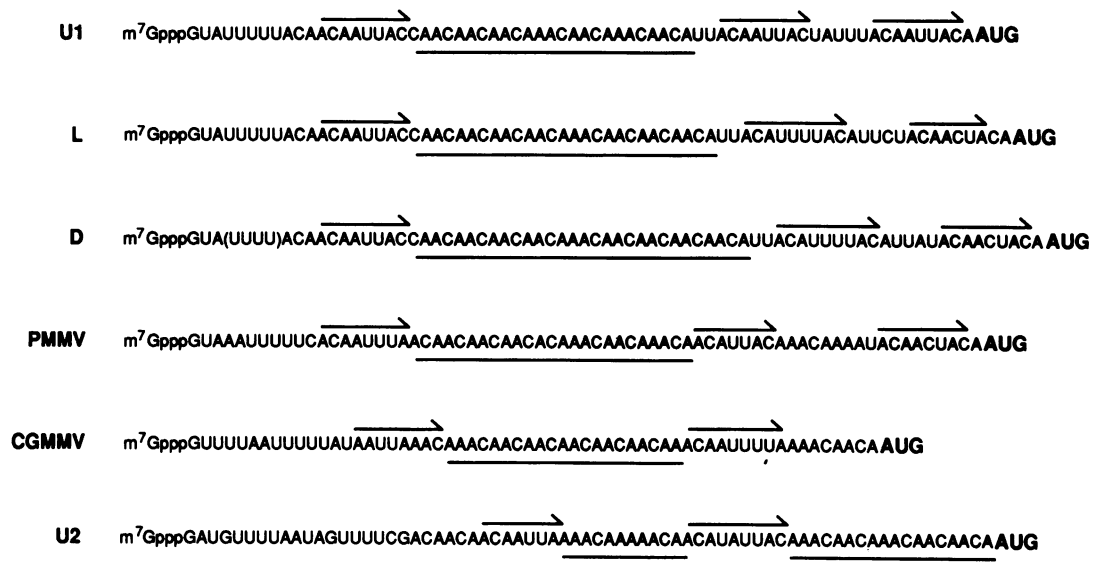

Figure 1. Phylogenetic analysis of the leader sequence from sequence TMV strains. Strains U1 (1), L (24), dahlemense (D)(25), pepper mild mottle virus (PMMV)(26), cucumber green mottle mosaic virus (CGMMV)(27), and U2 (28) were compared. Each leader sequence terminates in the initiation codon for the first open reading frame. Arrows delineate the eight base direct repeat, and the $(CAA)_n$ regions are underlined. The Ω used in this and previous studies is that leader from the U1 strain.

immediately downstream of the T7 transcriptional start site. As a result, all leader constructs generated contain the 5'-terminal seven base sequence of Ω. All constructs contain a *SalI* site between the test sequence and the start codon of the reporter gene. The context of the initiation codon, therefore, is identical for all constructs. The sequences of the leader constructs used in this study are illustrated in Fig. 2. The Ω leader construct (illustrated in the D series) is the wild type sequence with a *SalI* site present immediately before the initiation codon. The Luc and Ω-Luc mRNA constructs have been previously described (5).

All constructs were linearized downstream of the poly$(A)_{25}$ tract prior to mRNA synthesis. *In vitro* transcription was carried out as described (10) using 40 mM Tris-HCl pH 7.5, 60 mM $MgCl_2$, 20 mM spermidine, 100 mM NaCl, 100 μg/ml BSA, 0.5 mM each of ATP, CTP, UTP, plus 160 μM GTP, 1 mM m^7GpppG, 100 mM I dithiothreitol, 0.3 u/μl RNasin (Promega), and 0.5 u/μl T7 RNA polymerase (New England Biolabs). Under our transcription conditions, >95% of the mRNA is capped. The integrity and relative quantity of RNA were determined by formaldehyde-agarose gel electrophoresis as described (11). Equivalent amounts of each mRNA construct were used for electroporation.

Preparation and electroporation of plant protoplasts

Protoplasts were isolated from a carrot (RCWC) cell suspension by digestion with 0.25% CELF Cellulase (Worthington Biochemicals), 1% Cytolase M103S (Genencor), 0.05% Pectolyase Y23 (Seishin Pharmaceutical Co.), 0.5% BSA, 7 mM β-mercaptoethanol in isolation buffer (12 mM sodium acetate pH 5.8, 50 mM $CaCl_2$, 0.25 M mannitol) for 75 min. Protoplasts were washed once with isolation buffer, once with electroporation buffer (10 mM Hepes pH 7.2, 130 mM KCl, 10 mM NaCl, 4 mM $CaCl_2$, 0.2 M mannitol), and resuspended in electroporation buffer to a final concentration of 1.5×10^6 cells/ml. Two μg of each GUS mRNA construct was mixed with 0.8 ml of protoplasts immediately before electroporation (500 uF capacitance, 350 V) using an IBI GeneZapper. Each construct was tested in triplicate and the mean reported. The dose-response of RNA electroporation vs. GUS activity is linear through 30 μg of input RNA (3). Protoplasts were harvested following 20 hr incubation.

GUS and luciferase enzyme assay

Protoplasts, collected by centrifugation at 100×g, were sonicated in 0.5 ml of GUS assay buffer (50 mM sodium phosphate pH 7.0, 10 mM β-mercaptoethanol, 1 mM EDTA) and the cell debris pelleted. To an aliquot of the supernatant, 4-methylumbelliferyl-β-D-glucuronide was added to a final concentration of 1 mM. The reactions were incubated at 37°C, aliquots were taken at time intervals ranging from 60 to 180 min and added to 0.2 M sodium carbonate to terminate the reaction. Fluorescence was measured by excitation at 365 nm and emission at 455 nm in a TKO 100 DNA Fluorometer (Hoefer Scientific Inc.). GUS specific activity was determined as nanomoles of product (4-methylumbelliferone) produced per minute per milligram of protein. Endogenous GUS activity in carrot protoplasts is low (<25% of the lowest expressing constructs) and was subtracted from measured GUS activities. Protein was determined using the BioRad protein assay kit.

For luciferase assays, the cells were harvested and resuspended in luciferase assay buffer (25 mM Tricine pH 7.5, 15 mM $MgCl_2$, 7 mM β-mercaptoethanol, 1 mM ATP), sonicated for 5 sec, and the cell debris pelleted. Luciferase activity was measured following injection of 100 μl 0.5 mM luciferin with a Monolight 2010 Luminometer (Analytical Luminescence Laboratory).

RESULTS

Ω increases the rate of translation

We have shown that the enhancing effect of Ω is not due to increased transcript stability (3,5). To illustrate the effect of Ω on the rate of translation of a chimeric mRNA *in vivo*, capped luciferase (Luc) mRNA with or without Ω as the leader was

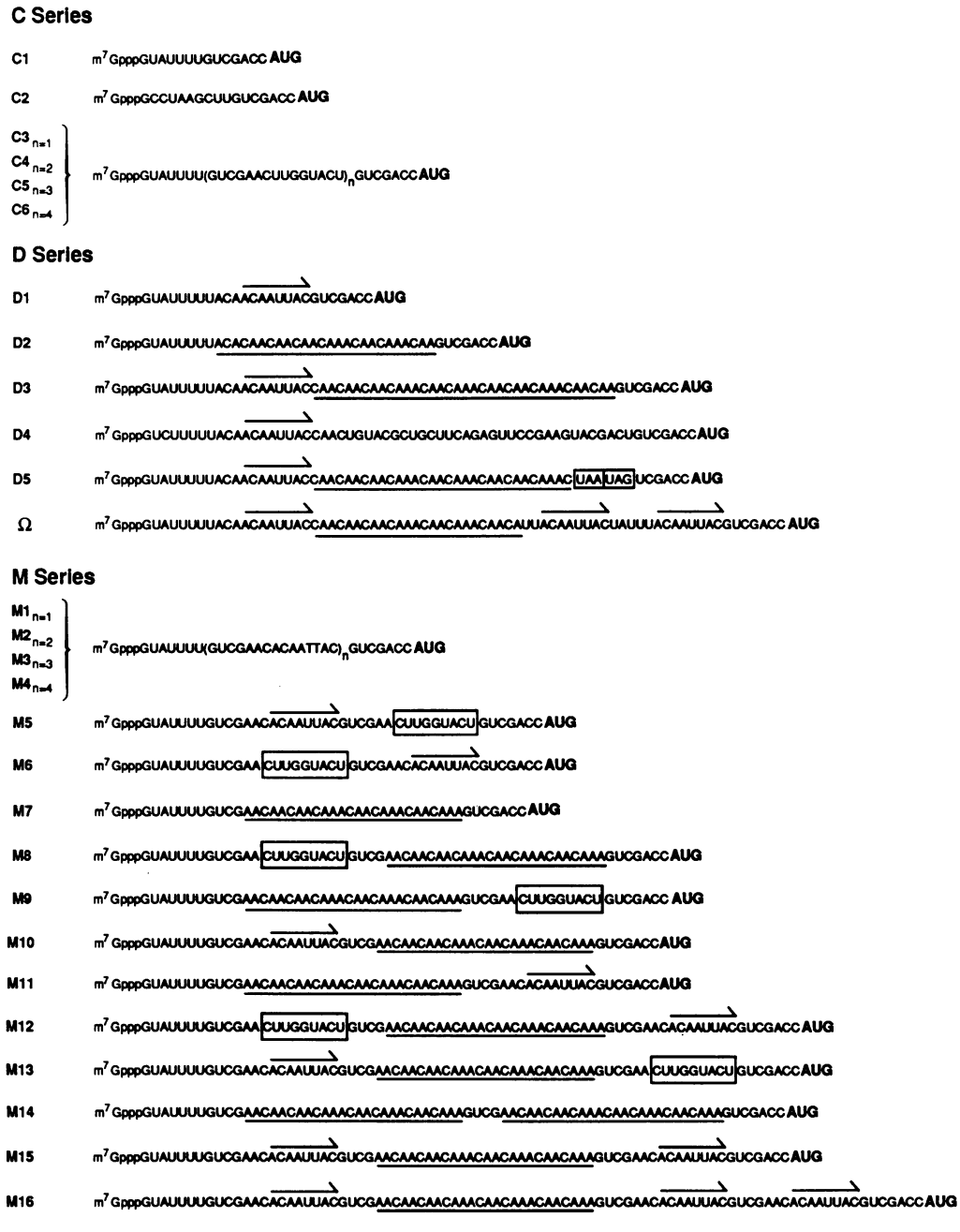

Figure 2. Leader constructs used in the analysis of leader length and Ω. Leader sequence is shown up to the initiation codon of the GUS reporter gene. The context around the initiation codon was identical for all constructs. An arrow indicates the eight base direct repeat from Ω; the $(CAA)_n$ region is underlined, and the random module used in the M series is indicated with a box. The two stop codons introduced in construct D5 are also boxed.

delivered to carrot protoplasts using electroporation for translation. The impact of Ω on the rate of translation was determined by following the appearance of luciferase enzyme over time. As luciferase protein is stable over the time frame of this analysis, its rate of appearance is directly related to its synthesis. Moreover, for mRNAs of equivalent stability, differences in its rate of synthesis is a consequence of differences in translational efficiency.

Although Luc mRNA with Ω or the control leader (C2, Fig. 2) were translationally active for approximately the same period of time, the translational efficiency of the Ω-Luc mRNA was markedly higher than that of the control (Fig. 3A). Products of translation can be detected as early as 3 min following delivery of Luc mRNA and even at the earliest time points, the Ω-containing construct is considerably more translationally active than the control (Fig. 3B). For instance, 6 min following mRNA delivery, the presence of Ω resulted in a >90-fold increase in translation. This kinetic analysis demonstrates that Ω enhances the rate of translation, however, we can not conclude from this data whether Ω enhances the rate of translation initiation, elengation, or termination, although initiation is considered the rate limiting step in translation (12).

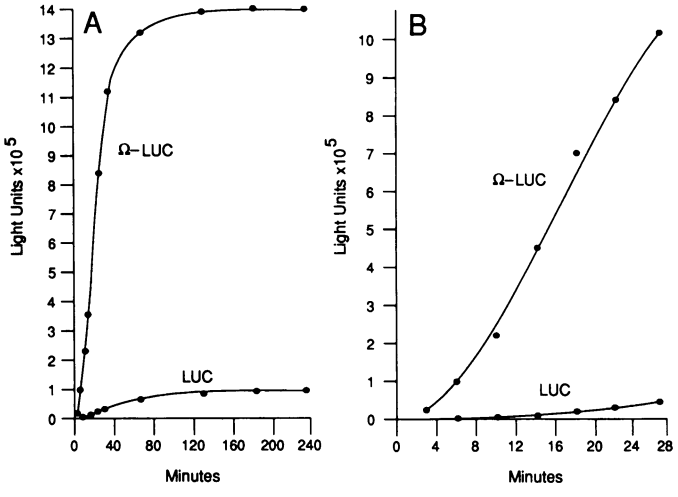

Figure 3. Impact of Ω on translational efficiency. Luc mRNA with Ω or a control leader (C2) was electroporated into tobacco protoplasts, aliquots of cells removed at specific time intervals, and assayed for luciferase activity. The kinetics of translation were followed as the rate of luciferase protein production (as measured by activity) over time. (A) Analysis of translation over the life of Luc mRNA. (B) The kinetics of translation during the first 28 min showing the effect of Ω shortly after commencement of translation.

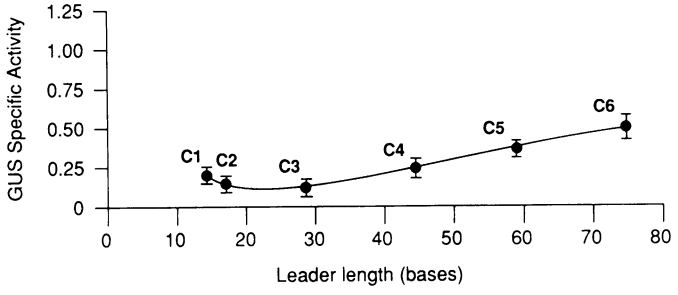

Figure 4. Effect of leader length on GUS expression in electroporated carrot protoplasts. *In vitro* synthesized C series mRNA constructs were translated in protoplasts, and the resulting GUS specific activity (nmoles/min/mg protein) was plotted as a function of leader length. Electroporation of each construct was carried out in triplicate. Standard error bars for the activity resulting from each construct are indicated.

Effect of leader length on translational efficiency

Leader length is one of the parameters known to influence translation in both animal cells (13) and in yeast (14). Previous work has compared Ω in an 84 base leader construct (67 bases from Ω and 17 bases from polylinker sequence) to a 17 base polylinker control leader (3,4,5). In this comparison, Ω confers a 30- to 35-fold translational advantage in carrot and tobacco protoplasts (3). We therefore examined the effect of leader length on translational efficiency in protoplasts to determine whether the difference in length between Ω and the control might account for the enhancement associated with Ω. A pair of complimentary oligonucleotides were designed to represent a 'random' 15 base sequence, $(5'\text{-GAACTTGGTACTGTC-}3')_n$ that could be used to generate a set of constructs (C series, Fig. 2) in which the leader varied in length (by multiples of 15 bases) but not in sequence. The two leader constructs (C1 and C2) used in the construction of the C series were included so that a range of leader lengths from 14 to 74 bases could be tested. These leader-GUS mRNA constructs were delivered to carrot protoplasts using electroporation and the impact of the leader on GUS expression measured.

Doubling the length of the leader from 14 bases (C1) to 29 (C3) bases did not increase GUS expression (Fig. 4). However, expression increased as a function of leader length from 44 bases up to 74 bases. Although the 17 base leader of C2 is longer than the 14 base leader of C1, the expression from C2 not significantly different than that from C1. It should be noted that the leader from C2 differs from the rest of the C series in that it contains the seven base 5′-terminal sequence, 5′-GCCUAAG-3′ instead of the 5′-terminal sequence of Ω (5′-GUAUUUU-3′) present in all other C series constructs. These data suggest that increasing leader length up to 44 bases does not increase translational efficiency in carrot protoplasts, however, further increases up to 74 bases do have a positive impact.

Identification of the core regulatory element in Ω using deletion analysis

Oligonucleotides, incorporating various deletions of the motifs present in Ω, were used to construct the deletion series (D-series, Fig. 2) of leader-GUS mRNA constructs. The Ω leader was also re-designed to delete the additional sequence present at the 5′ end of our previous Ω leader construct so that the new Ω leader (D-series, Fig. 2) contains a 5′-terminus identical to wild-type. These mRNAs were tested in carrot protoplasts using the C series constructs as controls for the leader-length effect. D1, representing the first 19 nucleotides of Ω, was translated to the same extent as C1 and C3 (Fig. 5). As D1 contains one copy of the eight base direct repeat, this motif alone is not responsible for the enhancement associated with Ω. The leader construct representing the $(CAA)_n$ region (D2) was expressed 3-fold higher than C4, the control construct with a leader of equivalent length. This observation suggests that the primary sequence and not simply the length of the D2 leader is responsible for the elevated expression. When the leader contained both the direct repeat and the $(CAA)_n$ motifs (D3), expression increased 6-fold over the appropriate leader-length control (C5). The level of expression of the wild-type Ω was also 6-fold greater than the equivalent length control construct, C6.

The fact that the enhancement afforded by D3 was equivalent to the wild type Ω (enhancement as measured using the leader-length control appropriate for each) suggested that D3 represents the core regulatory element in Ω responsible for translational enhancement. If this were indeed the case, then both motifs would be necessary for enhancement. It is possible, however, that the direct repeat motif may not function if positioned too close to the initiation codon, such as in D1. In this scenario, the $(CAA)_n$ region present in D3 might be serving as a spacer between the direct repeat and the initiation codon. To test this possibility, a construct equivalent in length to D3 was designed such that the $(CAA)_n$ region was replaced by a random sequence, resulting in D4. The level of expression from D4 was not elevated above that for the control (C5). The data from D4 demonstrates that the primary sequence of the $(CAA)_n$ region is critical for enhancement.

The AUU sequence within the 5′-copy of the direct repeat has been implicated as a second ribosomal binding site. Dipeptide analysis suggested that initiation occurs not only at the first AUG

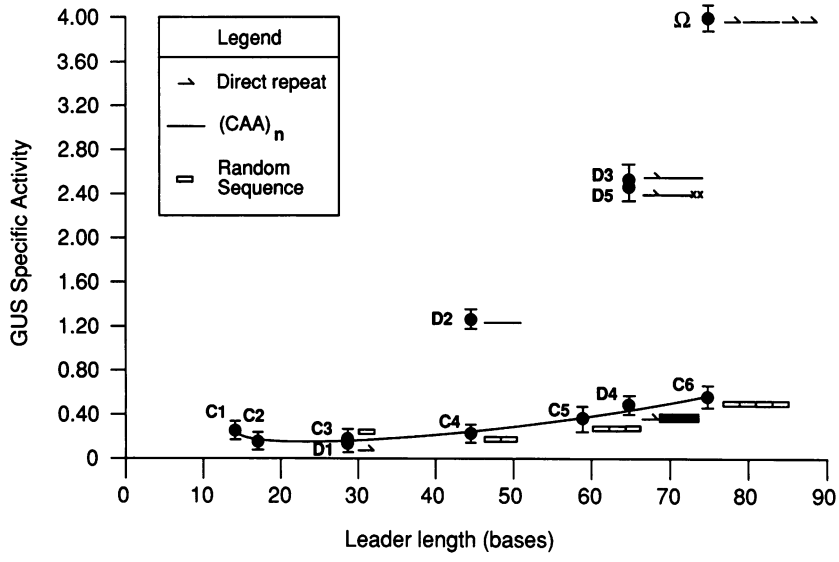

Figure 5. Deletion analysis of Ω. *In vitro*-synthesized D and C series mRNA constructs were electroporated into carrot protoplasts, and the resulting GUS specific activity (nmoles/min/mg protein) was plotted as a function of leader length. Leader constructs are illustrated schematically. The two stop codons present in D5 are schematically indicated by X's. A line drawn through the C series constructs delineates the effect of leader length on expression. Each construct was tested in triplicate electroporations. Standard error bars for the activity resulting from each construct are indicated.

immediately downstream of Ω, but at the AUU codon in the 5′-copy of the direct repeat (15). Mutation of the AUU to CUU had no effect on the enhancement afforded by the full-length Ω sequence (9). From this, it was concluded that any initiation occurring at the AUU codon of the 5′-copy of the direct repeat did not contribute significantly to the enhancement afforded by Ω. In this same mutant, however, the other two copies of the direct repeat remained unchanged, and may have masked the effect of the mutation on the 5′ copy of the direct repeat. To more rigorously test whether initiation at the AUU codon within the first direct repeat contributed to translational enhancement, 3 bases of the D3 leader were changed to introduce two stop codons in frame with both the AUU in the enhancing core element of D3 and the GUS initiation codon. This leader construct (D5) is equal in length to D3. Expression from D5 was equal to that from D3 (Fig. 5). Therefore, the presence of the stop codons had no effect on enhancement. These data demonstrate that if initiation occurs at the upstream AUU codon, it is not responsible for the enhancement associated with Ω.

Analysis of motif arrangement and functional redundancy within Ω

One conclusion arising from the initial deletion analysis (9) was that Ω may be functionally redundant. Moreover, the results from the D series of constructs suggest that Ω is composed of motifs specifically responsible for enhancing expression. An alternative to the deletional approach was necessary to investigate the functional redundancy and the role of arrangement of the motifs within Ω. Three sets of oligonucleotides were designed: a direct repeat module, a $(CAA)_n$ module, and a random-sequence module the same length as the direct repeat module. The design of the oligonucleotides enabled copies of the modules to be sequentially introduced into the leader region of the GUS gene in any order resulting in a module leader series (M series, Fig. 2) in which the number and relative position of the motifs was varied. mRNA constructs from the M series, selected D series constructs, and the C series constructs were then tested in carrot protoplasts.

One copy of the direct repeat module (M1) was equivalent in expression (Fig. 6) to the single copy of the direct repeat construct (D1) and the leader-length control construct (C3). Leader constructs composed of two modules, or of one copy of the $(CAA)_n$ module (which is twice as long as the direct repeat or random-sequence module), exhibited a greater range of expression. Two copies of the direct repeat module (M2) did not perform differently from the leader-length control (C4). The level of expression resulting from constructs with leaders composed of one copy each of the direct repeat and the random-sequence module in either configuration (M5 and M6) was not significantly different from C4 as well. Expression from the construct containing one copy of the $(CAA)_n$ module (M7), however, was statistically higher than C4. The results with M7 are in good agreement with the observations made for D2 in the previous experiment and suggest that the $(CAA)_n$ region is important in translational enhancement.

An even greater range in the level of expression was observed for leader constructs containing three copies of the direct repeat or the random-sequence module, or a combination of one copy of the $(CAA)_n$ module and one copy of either the direct repeat or random-sequence module. The leader construct containing three copies of the direct repeat module (M3) was expressed only marginally better than the control (C5). Constructs containing the $(CAA)_n$ and random-sequence module in either orientation (M8 and M9) exhibited elevated levels of expression and were in fact more highly expressed than M7, which contains the $(CAA)_n$ module alone. The increase in expression by the addition of the random-sequence module may be explained by the 15 base increase in leader length. The leader constructs containing one copy each of the $(CAA)_n$ and direct repeat modules in either configuration (M10 and M11) were significantly

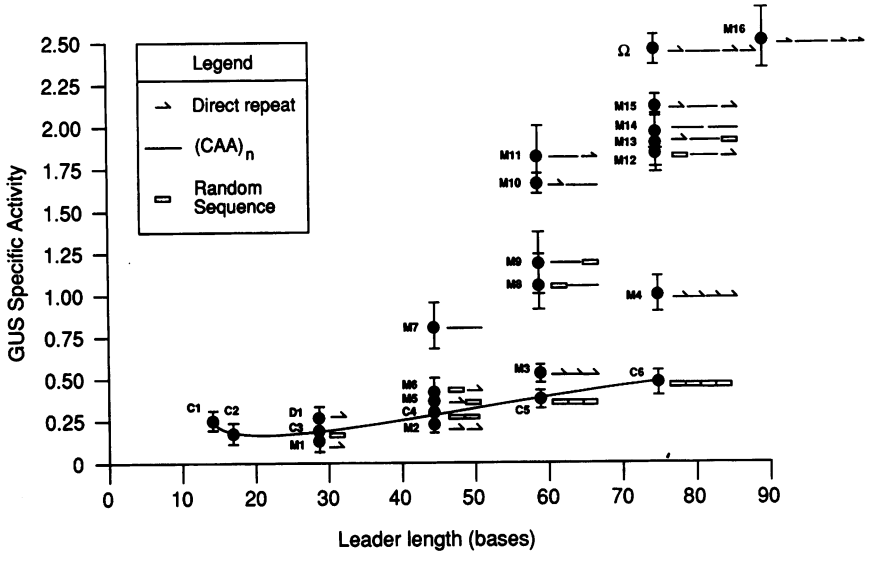

Figure 6. Effect of Ω motif arrangement and number in establishing translational efficiency. *In vitro*-synthesized M and C series mRNA constructs were electroporated into carrot protoplasts, and the resulting GUS specific activity (nmoles/min/mg protein) was plotted as a function of leader length. Leader constructs are illustrated schematically next to the data points. A line drawn through the C series constructs delineates the effect of leader length on expression. Each construct was tested in triplicate electroporations. Standard error bars for the activity resulting from each construct are indicated.

elevated in expression compared to both the control (C5) and M7. The increased expression from M10 and M11 was too great to be explained by the 15 base increase in leader length. The observations with M10 parallel that observed for D3, namely that the direct repeat motif and $(CAA)_n$ motif constitute the core regulatory element. Moreover, the results from M10 and M11 suggest that the regulation associated with the core element is relatively independent of the relative position of the two motifs.

Addition of a direct repeat module or random sequence module to M10 or M11 had only a small positive effect. Translation from these new constructs (M12, M13, M15) was higher than for the core element constructs, but the increase may be explained by the 15 base increase in leader length. Addition of two copies of the direct repeat module to M10, resulting in M16, increased expression possibly due to increased leader length. M16 contains the motif configuration of the wild type Ω sequence yet as a result of leader construction using modules, residual nucleotides from restriction sites are present. These extra nucleotides not only separate the motifs by four extra bases but also increased the length of the leader by 15 bases. The separation of the motifs in M16, however, had no impact on the ability of this leader to function as well as the wild type Ω construct. Moreover, the additional 15 bases present in M16 did not appreciably increase expression over Ω. The effect of increasing leader length may have a smaller effect for leaders >60 bases.

Functional redundancy was observed most clearly with constructs M4 and M14. Whereas expression from the leader constructs containing up to three copies of the direct repeat module paralleled the controls, when four copies were present, expression was significantly higher than the control, C6. M14, containing two copies of the $(CAA)_n$ module was as translationally active as the core element constructs, M10 and M11 as well as M12, M13, and M15. The observation from M14 suggests that the direct repeat in the core regulatory element can be functionally replaced by a second copy of the $(CAA)_n$ module. That all copies of the direct repeat motif present within Ω can be functionally replaced by $(CAA)_n$ is demonstrated by the fact that the level of expression from M14 is only 18% less than that for the wild type Ω construct.

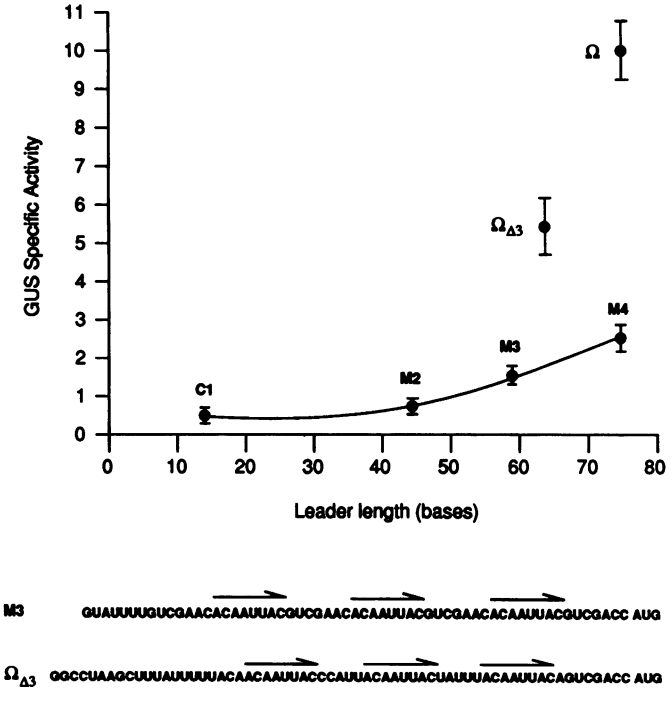

Figure 7. Analysis of direct repeat leader constructs in electroporated carrot protoplasts. Leader constructs with 0 (C1), 2 (M2), 3 (M3), or 4 (M4) copies of the direct repeat module were compared to the deletion mutant, $\Omega_{\Delta 3}$, previously described (9). Ω (D-series) was included as a positive control. Each construct was tested in triplicate electroporations. Standard error bars for the activity resulting from each construct are indicated.

The low level of enhancement from M3, which contains three copies of the direct repeat module contrasted with the level of enhancement observed for an Ω deletion mutant previously analyzed (9). In this mutant, $\Omega_{\Delta 3}$, the $(CAA)_n$ region was deleted, leaving a leader composed of the three direct repeats. Enhancement from this construct was 52% of the complete Ω construct (9). As the constructs were tested in tobacco mesophyll protoplasts whereas the present work was carried out in protoplasts made from carrot suspension cells, it was necessary to compare the three copy module leader (M3) directly with the deletion mutant leader $\Omega_{\Delta 3}$ in carrot protoplasts. The $\Omega_{\Delta 3}$ leader construct resulted in a level of expression that was 54% of Ω (Fig. 7) whereas expression from M3 was considerably less (14% of Ω). Both M3 and $\Omega_{\Delta 3}$ are approximately the same length (59 and 63 bases, respectively). The three direct repeats and the *Sal*I site account for 30 bases present in each leader, however, the remaining sequence is quite different between the two (Fig. 7). Moreover, the spacing between the direct repeats in M3 is greater than that in the $\Omega_{\Delta 3}$ leader. The context in which the direct repeat is placed or the spacing between repeats may influence their ability to fuunction. In this regard, the enhancement measured for M2, M3, and M4 should be viewed as a lower limit.

DISCUSSION

Using both deletion analysis of Ω and leaders constructed from modules, we have identified the motifs within Ω responsible for translational enhancement. We conclude that one copy each of the direct repeat and the $(CAA)_n$ motifs are necessary for wild-type enhancement of translation; together these constitute the core regulatory element. When either motif was replaced by random sequence, there was a loss of translational activity. Moreover, both Ω and the core regulatory element increased translational activity 6-fold over the respective controls. The $(CAA)_n$ motif, however, is more critical for function than the direct repeat. A leader containing a single copy of the $(CAA)_n$ motif increased translation 2.6-fold compared to a control construct of equal length. It is interesting to note that the TMV leader sequences illustrated in Fig. 1 all contain a $(CAA)_n$ motif. Enhancement was also observed when three, or to a greater extent, four copies of the direct repeat were present in a leader construct. However, the level of enhancement observed with the three direct module leader (M3) was not as great as that observed for the deletion mutant Ω_3. Either the context or the spacial relationship of the direct repeats may be important to their function. TMV leaders contain direct repeats that are, in many cases, variations of the direct repeat present in the U1 Ω investigated in this study. It will be of interest to determine the functional activity of these related direct repeats on translational enhancement.

Translation was influenced by leader length up to 74 bases, the longest leader tested. A 74 base leader construct was expressed 6-fold more highly than a 29 base leader construct. Interestingly, constructs with leaders 17 to 29 bases in length were translated less efficiently than when the leader was shorter or longer. In our previous work, Ω-containing constructs were compared to control leaders represented by C1 (6,7) or C2 (3,4,5). In the experiment illustrated in Fig. 6, Ω enhanced expression by 13 -fold over C2 in carrot protoplasts. With our leader length analysis, we conclude that approximately 2.4-fold of that level of enhancement by Ω can be attributed to leader length and 5.4-fold results from sequence-dependent enhancement by the core regulatory element present in Ω.

Although the conclusions from the D and M series of constructs concur, the leaders of these two series differ in one aspect, i.e., the direct repeat and $(CAA)_n$ motifs are contiguous in the D series as is the case in Ω but they are separated by up to seven bases in the M series. For the core element, the separation of the motifs may have had little effect on enhancement. Moreover, the position of the direct repeat with respect to the $(CAA)_n$ motif and initiation codon did not influence enhancement function. Ω itself contains a direct repeat both immediately upstream and downstream of the $(CAA)_n$ region. These data suggest that although both motifs are required for wild-type enhancement, there exists relative flexibility in both their spacing and relative position. One aspect of the Ω construct used in this study that we have not examined is the impact of the *Sal*I site that separates Ω from the initiation codon, which is not normally present in the wild type TMV leader. However, enhancement is observed for constructs in which Ω is separated by as much as 38 bases (16). A second consequence of the use of a *Sal*I site uptream to the AUG initiation codon was to alter the wild type context of the TMV AUG codon from ACAAUGGC to ACCAUGGC. The bases in the −3 and +4 positions (where the A of the AUG codon is +1) are considered to be the most critical for initiation codon selection in plants and animals (17, 18). The context of the TMV AUG initiation codon has been maintained at these positions. The one alteration from A to C at position −1 has been shown to be of little consequence for translation in plants (19).

Secondary structure within a leader can reduce translational efficiency (20, 21), whereas interactions between a leader and the downstream coding region have little effect on translation (22). The Ω-derived-leaders described in both the D and M series possess little secondary structure as predicted by the FOLD algorithm (23) yet there is a considerable range in expression. Moreover, the leaders of the leader-length control constructs were also designed to contain little potential for secondary structure. We conclude, therefore, that the core regulatory element within Ω functions actively to increase translational efficiency.

How the core regulatory element regulates translation remains to be elucidated. Ω neither increases transcript stability (3,5), nor determines initiation codon selection as it enhances equally translation of GUS mRNA containing a good or bad initiation codon context (7). However, in order to avoid any potential complications associated with changes in initiation codon context, the context surrounding the GUS and the Luc initiation codon was held constant in our series of constructs. It is possible that the core regulatory element serves as a binding site for a factor required for efficient initiation. Specific binding of a protein factor to Ω, and specifically to the $(CAA)_n$ motif, has been observed (R.Tanguay and D.Gallie, unpublished results). Characterization of the RNA-binding protein activity will be necessary to determine its role in translational enhancement.

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